THE EMBO JOURNAL

Manuscript EMBO-2012-83768

Structural basis for conformational switching and GTP loading of the large G protein atlastin

Laura J Byrnes, Avtar Singh, Kylan Szeto, Nicole M Benvin, John P O'Donnell, Warren R Zipfel and Holger Sondermann

Corresponding author: Holger Sondermann, Cornell University

Review timeline:

Submission date:	29 October 2012
Editorial Decision:	23 November 2012
Revision received:	14 December 2012
Accepted:	18 December 2012

Transaction Report:

(Note: With the exception of the correction of typographical or spelling errors that could be a source of ambiguity, letters and reports are not edited. The original formatting of letters and referee reports may not be reflected in this compilation.)

Editor: Thomas Schwarz-Romond

1st Editorial Decision 23 November 2012

Thank you for submitting your structural work on the atlastin reaction cycle for consideration to The EMBO Journal editorial office.

I received comments from two scientists that judge the provided experimental evidence very convincing and significant. At the same time however, some concerns remain on the final model proposed that demands a bit of further experimentation respective adjustments of the presentation before final assessment for eventual publication here.

As the suggestions particularly from ref#1 are explicit and constructive indeed, there is not much need to repeat them, but rather recommend expansion of the current dataset along these concise comments.

Please do note that The EMBO Journal considers only one round of major revisions with the ultimate decision solely depending on the quality and strength of the final version.

Please do not hesitate to get in touch in case of further questions (preferably via E-mail).

I am very much looking forward to a revised study in a timely manner and remain with best regards.

REFEREE REPORTS

Referee #1:

This is another great contribution of the Sondermann group describing an analysis of the atlastin reaction cycle and the conformational changes associated with nucleotide binding and hydrolysis. A thorough crystal structure analysis of atlastin in the GTP and GDP-AlF4-bound forms is followed by an elegant and comprehensive series of kinetic and functional experiments to deduce the structural framework for membrane remodelling at the ER. The study is a very important step forward since it answers a number of open questions related to the mechanisms of membrane remodelling in dynamin superfamily GTPases and in particular of atlastin. It is of great interest not only for structural biologist, but also for many biophysicists and biomedical researchers. While the experimental data are very convincing, the model how atlastin works in a nucleotide-dependent fashion is not so clear and might need some more work and discussion.

Major points:

The model appears in the middle of the manuscript (Fig. 6) while it would be more useful at the end incorporating also the results of Figs 7-9 (more details should be added). I also do not understand the indicated tether and release model. Why do the authors claim that GTP hydrolysis could take place in the monomeric state? This is completely contradictive to their structural data, showing that dimerization is linked to rearrangement of catalytic residues in the active site which is a prerequisite for GTP hydrolysis, as in all other dynamin-related GTPases.

Most if not all dynamin related GTPase described so far act in cis on the membrane, e.g. they oligomerise at the surface of one membrane and created membrane curvature required for membrane-remodelling. Atlastin is an exception since it has been proposed to act in trans, e.g. two atlastin monomers dimerise across two different membranes to tether them and mediate membrane fusion. Interestingly, the kinetic and structural data of this manuscript (unlike the previous two PNAS publications) appear to be more consistent with a model in which also atlastin initially dimerises in cis and not in trans. GTP-binding clearly favours the parallel conformation of the middle domain which is possible only in the same plane of the membrane. This is followed by GTP hydrolysis and the open conformation of the middle domain, which again allows GTP loading. These aspects of different models of atlastin action should be better worked out and discussed.

Figure 5 and Figure 8:
The kinetics for stalk rearrangements and G domain dimerization are faster for GTP versus GMPPNP which lead the authors to conclude that GTP hydrolysis could potentially occur in the context of a monomeric G domain (p20, middle).I find these results counterintuitive and confusing. First, according to Fig. 3B (GTPase assays), the kcat of atlastin is around 4 min-1, so GTP hydrolysis is actually slower compared to the kinetic changes of the FRET signal in the presence of GTP (range 30 min-1). Please clarify.
Second, GMPPNP is not always a good GTP analogue in functional assays since it has a different geometry at the gamma-phosphate position. Here, it appears to bind atlastin with similar affinity as GTP but it might not efficiently allow the switching to the parallel conformation of the middle domains. At least the experiments in Figure 5 should be repeated with GTP-gamma-S as an additional control which is more similar to GTP (the FRET signal might then appear as fast as for GTP). Also the slower kinetics for the R77A mutant do not necessarily support the hypothesis that GTP hydrolysis (versus GTP binding) promotes the formation of the parallel stalk arrangement - this mutant has most likely also some dimerization deficits. Finally, the apparent lack of cooperativity in GTPase assays is likely explained by the high affinity of atlastin's self-assembly in the presence of GTP (according to Fig. 3B and the GF data, the affinity for dimerization via the G domain is likely higher than 500 nM which is completely different in other dynamin superfamily members).

The data on GTP-loading in the open conformation are quite striking, but, as noted, also surprising. Is there a structural explanation why atlastin can bind GTP only in complex with the middle domain while GDP can be bound also in the absence of the middle domain? Can the authors estimate an affinity of the middle domain to the G domain (for example, by titrating the middle domain into a solution of the G domain containing saturating concentrations of mant-GMPPNP and following the increase of fluorescence). A biochemical characterization of atlastin-1 (1-446) M347E (nucleotide

binding, GTPase activity) would further support the conclusion that the G domain - middle domain contact is required for GTP loading.

Minor points:
GDP-AlF4: Is this really ALF4, with a square planar coordination of the fluorides? How were the GDP-AlF4 crystals set up (e.g. how was AlF4 set up, please add to the crystallization conditions)? The crystallization conditions appear to have a pH > 7.5, one of them contains phosphate. According to Reinstein and Schlichting, NSMB 6, 721-3, such pH favours formation of AlF3 rather than AlF4. If the electron density is more towards a tetrahedral coordination, it could be also GDP and phosphate. In the representations (for example, Fig. 3B), remove the link between the beta-phosphate and the Al ion. Is there a catalytic water molecule visible (if yes, show it and discuss)?

Fig. 2: The numbering of the crystal forms is very difficult to follow in the text. Why not use a more intuitive nomenclature, for example 'open', 'half closed' and 'closed' conformation (or something similar).

Fig. 3C GTPase assays: Better show rates than reaction velocities.

Fig. 5B: Include the appearance/disappearance of the three states in the graph and ideally a graphical reaction scheme showing how the three states interconvert.

Referee #2:

This paper reports the crytals structures of two new nucleotide-bound states of the soluble N-terminal region of the GTPase atlastin. In combination with previously solved structures the new structures reveal nucleotide-dependent changes in switch regions within the G domain, changes in the orientation of the middle/stalk domain relative to the G domain and changes in the modes of interaction of middle domains within an atlastin dimer that give rise to three forms/conformational states of the atlastin dimers. The mechanism of GTP hydrolysis is revealed, for the first time, by the crystal structure of the activated atlastin bound to GDP•AlF4-. A catalytic arginine finger residue (R77) is identified structurally and its essential role in hydrolysis confirmed biochemically.

FRET-based assays are used to measure the nucleotide-dependence and relative kinetics of middle domain and G domain dimerization. These results, which show temporally coincident and GTPase-interdependent conformational changes, favor a new models for a coordinated tethering and release of adjacent membranes as opposed to previously proposed modesl for sequential GTP binding, conformational changes and then hydrolysis.

Finally, the authors provide strong evidence that middle domain interactions with the G domain influence its ability to bind GTP and dimerize.

The results presented are interesting and well supported by the data. They provide new and important mechanistic information to help develop models for atlastin-mediated membrane fission. Of course the final 'tether and release' model that is presented still falls far short of linking these conformational changes and states to membrane fusion. Much work remains to be done.

Minor concerns:

The description of the SEC-MALS data lacks sufficient detail to understand the data presented. What to the dimer and monomer lines mean? I understand that the dimer elutes earlier than the monomer, but neither surpasses these lines? What do the black 'squiggles' on these graphs mean? What is the significance of their slopes? What species do the three peaks in Figure 9 D (G + M) correspond to (I assume the most rapidly eluting peak is M alone?

1st Revision - authors' response 14 December 2012

We would like to thank the reviewers for the positive evaluation and constructive feedback. As outlined below, we have addressed all points either by providing additional clarification and discussions, or by additional experiments.

Referee #1:

1. Major: The model appears in the middle of the manuscript (Fig. 6) while it would be more useful at the end incorporating also the results of Figs 7-9 (more details should be added). I also do not understand the indicated tether and release model. Why do the authors claim that GTP hydrolysis could take place in the monomeric state? This is completely contradictive to their structural data, showing that dimerization is linked to rearrangement of catalytic residues in the active site, which is a prerequisite for GTP hydrolysis, as in all other dynamin-related GTPases.

As suggested, we moved the model to the end, following the experimental data and incorporating all data points, and only show the one that is most consistent with our data. While we acknowledge that hydrolysis within a monomer is unusual within the dynamin family of G proteins, we would like to stress that the new structures identified an intramolecular arginine finger, indicating that the entire catalytic machinery is present in one protomer. Our structures merely show that dimerization occurs at some point after GTP binding, while the kinetic data (G and middle domain FRET) are suggestive of dimerization post GTP hydrolysis. In contrast, other dynamin-like proteins rely more heavily on dimerization to construct a catalytically active state. Hence, we don't see a contradiction between our model and the data, which actually support our model.

2. Major: Most if not all dynamin related GTPase described so far act in cis on the membrane, e.g. they oligomerise at the surface of one membrane and created membrane curvature required for membrane-remodelling. Atlastin is an exception since it has been proposed to act in trans, e.g. two atlastin monomers dimerise across two different membranes to tether them and mediate membrane fusion. Interestingly, the kinetic and structural data of this manuscript (unlike the previous two PNAS publications) appear to be more consistent with a model in which also atlastin initially dimerises in cis and not in trans. GTP-binding clearly favours the parallel conformation of the middle domain which is possible only in the same plane of the membrane. This is followed by GTP hydrolysis and the open conformation of the middle domain, which again allows GTP loading. These aspects of different models of atlastin action should be better worked out and discussed.

We respectfully disagree with the reviewer that our new data are more consistent with a model that atlastin dimers form on the same membrane (cis mechanism) for curvature generation. Given the N-terminal cytosolic module used in our studies is connected to the transmembrane segments via a short but flexible linker, the parallel dimer could still form in trans. The kinetic data merely indicate that G and middle domain dimerization occur at the same time scales that are similar to nucleotide binding (and hydrolysis). We prefer an interpretation that the G and middle domains function as tethers, more similar to SNARE-mediated fusion events. In this model, Atlastin's N-terminal domains would bring two membranes into close proximity and GTP binding, hydrolysis and middle domain release from the G domain serve as regulators or timers for the reaction cycle.

Based on our data, we also disagree with the statement that "GTP binding clearly favors the parallel conformation". As our kinetics data show, it takes quite some time for the middle domains to dimerize in the absence of GTP hydrolysis. Our data also indicate that the tight-parallel dimer conformation follows GTP hydrolysis (unlike the interpretation provided by the reviewer). As such, the new crystal structures are likely depicting the post-hydrolysis state.

We admit that we cannot unambiguously distinguish between our model and that suggested by the reviewer. However, we would like to point out that curvature generation is most likely achieved by the action of atlastin's amphipathic, C-terminal helix (its importance for fusion has been demonstrated by the McNew and Rapoport groups; references Moss et al, 2011; Stefano et al., 2011; Liu et al., 2012 in our manuscript). Finally, it is important to keep in mind that fission and fusion may require slightly different mechanisms. While fission originates from one membrane, classical fusion would still rely on bringing two membranes that will fuse into close proximity. This has been discussed in the revised manuscript (page 23; final paragraph of the conclusion section).

3. Major: Figure 5 and Figure 8: The kinetics for stalk rearrangements and G domain dimerization are faster for GTP versus GMPPNP which lead the authors to conclude that GTP hydrolysis could potentially occur in the context of a monomeric G domain (p20, middle). I find these results

counterintuitive and confusing. First, according to Fig. 3B (GTPase assays), the kcat of atlastin is around 4 min-1, so GTP hydrolysis is actually slower compared to the kinetic changes of the FRET signal in the presence of GTP (range 30 min-1). Please clarify.

We thank the reviewer for pointing out this apparent discrepancy between rates. The GTPase assays shown in Figure 3B are continuous measurements of phosphate release, and not hydrolysis per se. We predict that phosphate release is the rate-limiting step in our system, obscuring the true rate of GTP hydrolysis. Accordingly, we changed the labeling of the y-axis in Figure 3B, which now reads "Apparent GTPase Activity", and we have discussed this point on page 21.

4. Major: Second, GMPPNP is not always a good GTP analogue in functional assays since it has a different geometry at the gamma-phosphate position. Here, it appears to bind atlastin with similar affinity as GTP but it might not efficiently allow the switching to the parallel conformation of the middle domains. At least the experiments in Figure 5 should be repeated with GTP-gamma-S as an additional control, which is more similar to GTP (the FRET signal might then appear as fast as for GTP). Also the slower kinetics for the R77A mutant do not necessarily support the hypothesis that GTP hydrolysis (versus GTP binding) promotes the formation of the parallel stalk arrangement - this mutant has most likely also some dimerization deficits. Finally, the apparent lack of cooperativity in GTPase assays is likely explained by the high affinity of atlastin's self-assembly in the presence of GTP (according to Fig. 3B and the GF data, the affinity for dimerization via the G domain is likely higher than 500 nM which is completely different in other dynamin superfamily members).

We are aware of the limitation of GppNHp and other nucleotide analogs. As suggested by the reviewer, we included data using GTPγS in the revised manuscript (Figure S4A; time-resolved FRET G and middle domain). Unlike the reviewer's prediction, dimerization kinetics are similar to that of GppNHP and GDP•AlF_x (and when the R77A mutant is used), strongly arguing that GTP hydrolysis drives the conformational change and dimerization.

Regarding the R77A mutant, at least at the conditions tested, no apparent effect of the mutation on atlastin dimerization could be detected in the presence of GppNHp (Figure 3C). In addition, our FRET data clearly show a GTP hydrolysis-dependence for fast dimerization of both, the G and middle domains, which is also consistent with catalysis within a monomer. The strong dimerization of atlastin's N-terminal domains is indeed unusual, and could point to a mechanism (e.g. a function as tight tethers) discrete from the mechanisms employed by dynamin. Certainly, each data point alone may not be convincing but we feel that their combination and integration provide fairly compelling evidence for the proposed model.

5. Major: The data on GTP-loading in the open conformation are quite striking, but, as noted, also surprising. Is there a structural explanation why atlastin can bind GTP only in complex with the middle domain while GDP can be bound also in the absence of the middle domain? Can the authors estimate an affinity of the middle domain to the G domain (for example, by titrating the middle domain into a solution of the G domain containing saturating concentrations of mant-GMPPNP and following the increase of fluorescence). A biochemical characterization of atlastin-1 (1-446) M347E (nucleotide binding, GTPase activity) would further support the conclusion that the G domain - middle domain contact is required for GTP loading.

We favor a model in which the middle domain allosterically effects the conformation and/or dynamics of the switch regions, which is suggested by the structural comparison described in the manuscript. As discussed there, the differences in the structures and the proximity of the middle domain-docking site to the γ-phosphate moiety are in line with such an interpretation. As requested by the reviewer, we provide an estimate for the affinity between the middle and G domain in the revised manuscript (Figure S4B; also see page 20). Importantly, in the context of the construct comprising the G and middle domains, the middle domain would be present at extremely high local concentration, since it is covalently attached to the G domain via a short linker. We also tested the M347E mutant in the G and middle domain-containing protein, and observed a decreased affinity for nucleotide and reduced GTPase activity (Figure S4C; Table 1), consistent with the proposed model. All these new data points are discussed in the revised manuscript.

6. Minor: GDP-AlF4: Is this really ALF4, with a square planar coordination of the fluorides? How were the GDP-AlF4 crystals set up (e.g. how was AlF4 set up, please add to the crystallization conditions)? The crystallization conditions appear to have a pH > 7.5, one of them contains phosphate. According to Reinstein and Schlichting, NSMB 6, 721-3, such pH favours formation of AlF3 rather than AlF4. If the electron density is more towards a tetrahedral coordination, it could

be also GDP and phosphate. In the representations (for example, Fig. 3B), remove the link between the beta-phosphate and the Al ion. Is there a catalytic water molecule visible (if yes, show it and discuss)?

We added the additional details as requested. While one condition has phosphate, the other one does not. Refinement statistics and observed densities are also consistent with AlF_4. Thus far, we have not obtained crystals with GTP or GDP+phosphate (except for the ones described in our PNAS paper, which turned out to be GDP-bound states). Crystallization under these conditions is dependent on the nucleotide species added, supporting the presence of AlF_x (AlF_4^- based on the observed geometry and refinement). As requested, we removed the link between the beta-phosphate and Al ion, and added the discussion of catalytic waters (see Figure legend of Figure 3B).

7. Minor: Fig. 2: The numbering of the crystal forms is very difficult to follow in the text. Why not use a more intuitive nomenclature, for example 'open', 'half closed' and 'closed' conformation (or something similar).

We are sensitive to this issue. Unfortunately, since we need to distinguish between monomeric and dimeric states/conformations, the suggested nomenclature is not sufficient. We attempted to come up with a more descriptive nomenclature in the revised manuscript and have introduced more contextual descriptions, which we hope will improve readability.

8. Minor: Fig. 3B GTPase assays: Better show rates than reaction velocities.

We introduced the rates in the revised manuscript on page 9.

9. Minor: Fig. 5B: Include the appearance/disappearance of the three states in the graph and ideally a graphical reaction scheme showing how the three states interconvert.

We introduced the model reaction scheme in the revised Figure 5B.

Referee #2:

Minor concerns: The description of the SEC-MALS data lacks sufficient detail to understand the data presented. What to the dimer and monomer lines mean? I understand that the dimer elutes earlier than the monomer, but neither surpasses these lines? What do the black 'squiggles' on these graphs mean? What is the significance of their slopes? What species do the three peaks in Figure 9 D (G + M) correspond to (I assume the most rapidly eluting peak is M alone?

We thank the reviewer for pointing this out, and apologize for the confusion. As clarification, we extended the figure legend for Figure 3C, where MALS data is discussed first.

